The EF-P aminoacylation pathway may be a potential new target for antimicrobial drugs

Honors Research Thesis

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Abstract

Antibiotic resistance is a growing problem in both the developing world and industrialized nations. Bacterial infections are no longer cleared with a single round of antibiotics. The problem could be combated by discovering new pathways to target with drug treatment. One such possible pathway involves elongation factor P (EF-P), a bacterial protein involved in the regulation of antibiotic resistance and survival in other cellular stress. The modification of EF-P with (R)-β-Lysine by the lysyl-tRNA synthetase paralog PoxA affects protein synthesis in the ribosome by relieving stalling during translation of polyproline stretches (7). In PoxA deletion strains, EF-P is not modified decreasing cell replication rate, cell survival to stressful conditions and virulence of *Salmonella enterica*. By analyzing the contact surface between EF-P and PoxA and comparing it to the complex of a tRNA and an aminoacyl-tRNA synthetase, we were able to identify the novel interactions that could be a potential drug target. Most of the conserved interactions in the EF-P and PoxA complex correspond to the acceptor arm of the tRNA, but many of the contacts are unique. Through mutating amino acids involved in polar contacts between PoxA and EF-P and replacing them with alanine through site directed mutagenesis, it was determined which contacts (both novel and conserved) are important for EF-P recognition. This was measured by analyzing the aminoacylation kinetics using either EF-P or PoxA mutants. Our results suggest that recognition of EF-P by PoxA is mainly accomplished through binding of conserved amino acids that resemble the acceptor stem of a tRNA, but the arginine 235 contact may provide a target for antibiotic development.

Introduction

Antibiotics have been in use for over 70 years and although their benefits are clear, the over and misuse of antimicrobials has led to the spread of multi-drug resistant pathogens (1,2). Pathogens are increasingly resistant to the available antibiotics, which is complicated by hampered pharmaceutical development(2,3). Thus, patients endure greater costs and longer hospital stays (3). The demand for new antibiotics is clear, but scientists face the problem of discovering a new, effective pathway to target. Since most of the obvious antibiotics, like penicillins, which disrupt external features like cell walls, have already been produced, scientists instead focus on new targets for drugs (2).

Elongation factor P (EF-P) is a bacterial protein that is found in all bacteria and is an ortholog of the eukaryotic and archaeal initiation factor 5a. It is required for pathways involved in antibiotic resistance and motility. It is additionally important in survival under stress conditions created by S-nitroso-glutathione (GNSO) that mimics the nitric oxide burst seen in phagocytes (4). EF-P, a structural and functional mimic of tRNA, is aminoacylated *in vivo* by the protein PoxA (previously named GenX or YjeA) with (R)-β-Lysine at the K34 modification site (5). Modified EF-P has been shown to prevent ribosome stalling during the translation of sequential proline codons by restoring the activity of the ribosome that is disrupted by the polyproline stretch (6). In the absence of EF-P, proteins with a series of three or more prolines are truncated during translation (7). The modification of EF-P with (R)-β-Lysine is essential for the high efficiency of this function (6). Proteins with a three or more consecutive prolines make up about 7% of the *Escherichia coli* genome and include proteins such as the flagellar proteins (FlhC, FliF, Flk), explaining EF-P's effect on cellular motility, TonB, a protein that provides

energy for cellular transporters, and EspF which is an *E.coli* virulence protein of enterohaemorrhagic strains (7).

PoxA, that aminoacylates EF-P with (R)-β-Lysine, is a paralog of the catalytic domain of lysyl-tRNA synthetase(LysRS)(8). As both PoxA and EF-P are essential for determining virulence, motility and survival during cellular stress, we propose the PoxA EF-P aminoacylation reaction as a potential target for antibiotic development.

In order to determine if there are any sites of PoxA where EF-P binds different than tRNA binds to aaRS, the contacts of EF-P and PoxA were identified and the polar amino acid contacts of PoxA were mutated to alanine. The effect of the mutations on the aminoacylation of EF-P was measured and revealed that most of the important interactions between PoxA and EF-P are equivalent to the ones that bind to the acceptor stem of tRNA. One of these contacts that is important for EF-P recognition has no corresponding contact in either the *Saccharomyces cerevisiae* aspartyl-tRNA synthetase (AspRS) or the Human lysl-tRNA synthetase (LysRS), suggesting a potential antimicrobial target.

Results

Only part of the AspRS/tRNA^{Asp} contacts are conserved on EF-P/PoxA complex

To identify the contacts that evolved to recognize protein instead of RNA and which amino acids were conserved, the PoxA EF-P contacts were compared to the aspartyl-tRNA synthetase/tRNA^{Asp}(AspRS/tRNA^{Asp}) complex. An AspRS crystal structure was used instead of LysRS because the tRNA in complex with LysRS is not well resolved. Because AspRS and LysRS are both sub-class 2b synthetases and have structural homology, AspRS is a logical substitute. In comparing to the AspRS/tRNA^{Asp} system, many of the contacts between EF-P and

PoxA are unique, most likely due to the lack of the anticodon binding domain in PoxA which is responsible for most of tRNA binding energy by aminoacyl-tRNA synthetases (H52, D177, N180, E185, Q193,S218, R235, E244) (Figure 1). There are some contacts that are highly conserved and those amino acids are the ones that contact the acceptor arm (E103, R106, H108).

Analysis of the exchange of each of the EF-P/PoxA contacts

In order to analyze the recognition of EF-P by PoxA, 11 mutants were constructed that exchanged to alanine amino acids making direct polar contacts. To compare the effects of the mutations and therefore the importance of the contacts, the aminoacylation of EF-P with ^{14}C α-lysine was measured for all mutants and compared to wild type PoxA (Table 1). Some mutations had little or no effect on aminoacylation (Q193A, S218A, R106A, D177A), while some mutations reduced aminoacylation by half (H52A, N180A, E185A). The mutations that completely inhibit aminoacylation of EF-P are the contacts that interact with EF-P's structural mimic of the acceptor stem of tRNA (E103, H108, R235).

These results were confirmed by aminoacylation experiments performed with the natural substrate of PoxA ((R)-β-Lysine). Since we lack access to radioactive (R)-β-Lysine , EF-P was tagged with a cAMP dependent protein kinase A (pka) site to make ^{32}P radiolabelled EF-P. Because aminoacylated EF-P and non-aminoacylated EF-P have different isoelectric focusing points, they were separated on an isoelectric focusing gel with a 4.5 to 5.4 pH gradient. The radiolabelled EF-P allows for quantification by phosphor imaging, and this phosphorylation does not interfere with the aminoacylation reaction. These experiments confirmed the results of the ^{14}C α-Lysine experiments (Table 1)(9).

Comparing the important contacts to AspRS/tRNA^{Asp}

Only one of the polar contacts studied has a drastic effect on aminoacylation and has no corresponding contact in the AspRS/tRNA^{Asp} system (R235). Although this residue is conserved in the LysRS synthetase (R485), the crystal structure superimposed upon the AspRS/tRNA^{Asp} complex of *S. cerevisiae* indicates that it is not a contacting the tRNA (Figure 2). The shortest distance between R485 and the tRNA is 9.14 Å.

In order to confirm that these polar contacts are important for recognition by PoxA, the corresponding amino acids on the acceptor stem mimic of EF-P (F29, K31, G33), or the identity elements, were mutated to alanine. The exchange of any of these residues almost completely prevents aminoacylation by PoxA indicating that these residues are all individually important for recognition (Figure 3).

Discussion

Our results show that recognition of EF-P by PoxA is mainly accomplished through binding of amino acids that resemble the acceptor stem of a tRNA. Most of the contacts are novel compared to the AspRS/tRNA^{Asp} system; nevertheless, the contacts that are important for PoxA recognition are both previously existing contacts that have an enhanced binding importance(E103, H108) and one of the new contacts (R235), all of which are interacting with EF-P identity elements. These identity elements correspond to the CCA of the acceptor stem of a tRNA (Figure 4).

EF-P PoxA contacts were compared to the AspRS/tRNA^{Asp} system, to identify a potential drug target. Novel contacts would be the most ideal for a drug that targets bacteria to avoid host cell damage. Since PoxA evolved from a lysyl-tRNA synthetase, the potential for a PoxA inhibitor to also block host LysRS must be considered. The EF-P and PoxA contacts were

compared to the sequence of human AspRS, LysRS and AsnRS and only LysRS conserves the corresponding R235 residue (Figure 5). In *S. cerevisiae*, the corresponding amino acid is a serine, rather than arginine, but the crystal structure of the AspRS/tRNA^{Asp}(8,) shows that serine avoids tRNA contact (Figure 2). This position on PoxA is a possible target for blocking aminoacylation of EF-P in pathogenic bacteria that would likely not interfere with aminoacylation by Human LysRS. Further experiments need to be done to confirm this.

Scientists are focusing on finding new targets for antibiotic development to combat resistance. Targets can be easily identified with genetic screening, but the actual development process takes years (10). Thus, the many targets that have been identified, like EF-P, require assays to determine the site, compound and efficiency of an inhibitor. Understanding the interactions between PoxA and EF-P helps to identify the residues that are important for recognition and is the first step in finding a binding site of a PoxA inhibitor.

Materials and Methods

Bioinformatic Analysis

The contact surface between PoxA and EF-P was analyzed using the program PDBe/PISA[5] with the crystal structure of the complex (pdb3A5Z). Polar contacts previously described were also included in structure analysis(8). Images were made with The PyMOL Molecular Graphics System (Version 1.2.0.3 Schrödinger, LLC) and Swiss-PdbViewer (Version 4.0.4). Sequence alignment was performed using COBALT.

Mutagenesis of PoxA and EF-P

Both EF-P and PoxA mutations were performed by the Stratagene QuickChange Site Directed Mutagenesis protocol as suggested by the manufacturer (Stratagene). Briefly, EF-P pTYB11 or PoxA pTYB11 were amplified with primers of tables 3 or 4. Using a thermocycler with a melting temperature of 95^{o}C and an annealing temperature of 55^{o}C, the primers annealed to the plasmid at the site of mutation and *pfu* DNA polymerase extended the primer, replicating the DNA completely, leaving a nick at the end. After digestion of the methylated (unmutated DNA) with DpnI, the amplified DNA was transformed in *E.coli* XL1Blue cells, which repaired both nicks that remained in the plasmid.

Plasmid sequences were confirmed through sequencing at the Plant-Microbe Genomics Facility (PMGF) at The Ohio State University and then transformed in *E. coli* BL21 XJB (DE3) cells for overexpression in autoinduction media containing metal mix (50 nM $FeCl_3$, 20 nM $CaCl_2$, 10 nM $MnCl_2$, 2 nM $CuCl_2$, 2 nM $NiCl_2$, 2 nM H_3BO_3), NPS (50mM Na_2HPO_4, 50 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$), 5052 (0.025% glycerol, 0.0025% glucose and 0.01% lactose) and 1mM $MgSO_4$).

Purification of EF-P, PoxA and mutant proteins

Purification of all proteins was performed using chitin columns as previously described[4] Briefly, cells were resuspended in buffer A (50 mM Tris HCl pH 8.0, 500 mM NaCl and 10% glycerol) plus 0.05% triton X-100 and lysed by sonication with 0.75 mM PMSF. Cell debris was eliminated by centrifugation (70 min at 75600g) and the supernatant loaded to a chitin column. The column was washed with 5 volumes of buffer A plus 0.05% triton X-100 and then with 25 volumes of buffer A. Finally the column was washed with 2 volumes of buffer A plus 100 mM DTT to stimulate digestion and incubated overnight at 4ºC. The protein was eluted with buffer A

and concentrated using filter centrifugation unit MWCO of 3 KDa. Then the protein was dialyzed against dialysis buffer (25 mM Tris HCl pH 8.0, 150 mM NaCl and 4 mM 2-mercaptoethanol) plus 50 % glycerol and stored at -20ºC. EF-P used for k_{cat}/K_M determinations was further purified through anion exchange using a Resource Q column. Protein was loaded on the column and washed with 10 volumes of Buffer 1 (25 mM Tris HCl pH8, 50 mM NaCl and 2 mM 2-mercaptoethanol), then with additional 6 volumes of 10% Buffer 2 (25 mM Tris HCl pH8, 1 M NaCl and 2 mM 2-mercaptoethanol) in Buffer 1. Finally the protein was eluted in a linear gradient to 14% Buffer 2 in Buffer 1, concentrated and dialyzed against dialysis buffer plus 20% glycerol. The protein was then stored aliquoted at -80ºC. All proteins were quantified in 6M guanidinium HCl using absorbance 280 and a molar extinction coefficient of 24750 M^{-1} cm^{-1} (EF-P) or 29870 M^{-1} cm^{-1} (PoxA) which were calculated using EMBOSS (10).

Aminoacylation with ^{14}C α-Lysine

In order to obtain relative activity data, EF-P aminoacylation by the PoxA mutants was measured with ^{14}C α-Lysine. Although α-Lysine is not the natural substrate of PoxA[ref], EF-P is still aminoacylated but at a much higher K_m. This reaction was performed in a mixture containing 100mM glycine pH 9.0, 30 mM KCl, 10 mM $MgCl_2$, 8 mM ATP pH 7.0, 3 mM β-mercaptoethanol, 8.36 U/mL pyrophosphotase and 0.75μM or 10 μM EFP. At defined time points, aliquots were taken and either stopped by mixing with SDS-PAGE loading buffer or precipitated on 5% trichloroacetic acid saturated 3MM Whatman paper. Papers were washed at RT with 5% tichloroacetic acid 3 times, for five minutes each, once with ethanol and dried at ~85 ºC. The ^{14}C aminoacylated EF-P was quantified using scintilation counting. Aliquotes of

the reaction were separated in a SDS-PAGE and ^{14}C α-Lysyl EFP was detected by phosphor imaging.

Figures

Figure 1: Contact surface in EF-P/PoxA and AspRS/tRNA^{Asp} complexes. Image corresponds to a superposition of AspRS and PoxA from pdb files 1asy and 3a5z. A) AspRS in blue, PoxA in magenta. B) EF-P in light green and tRNA^{Asp} light orange.

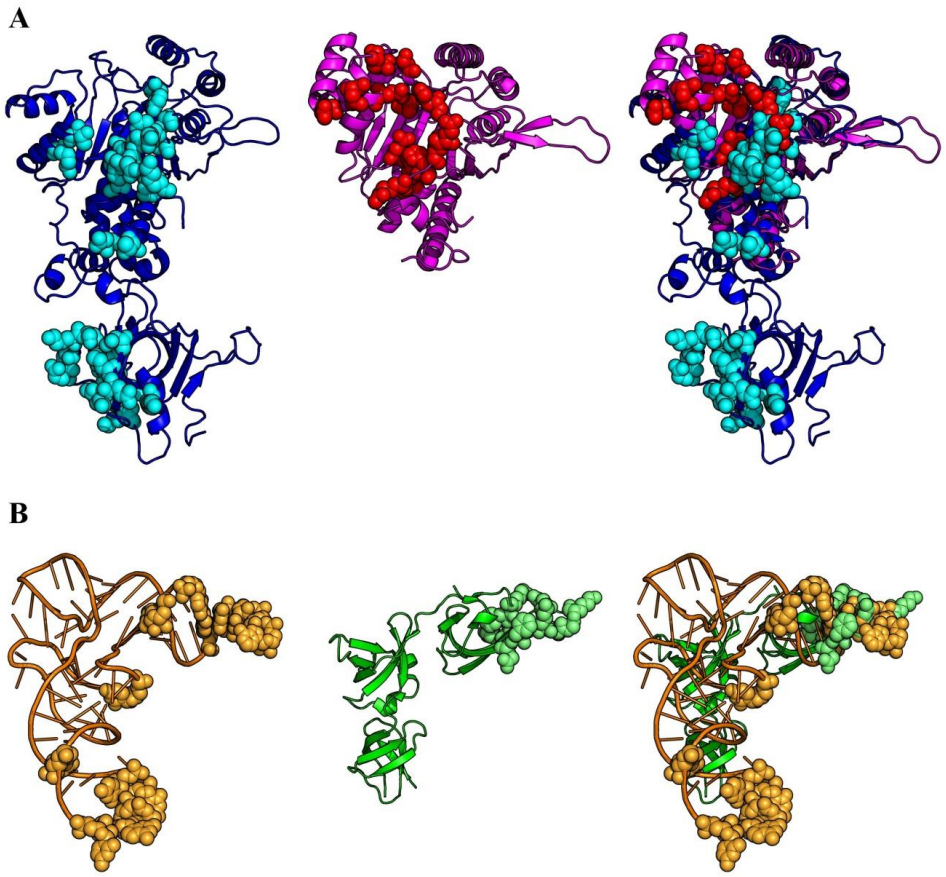

Table 1: Effect of mutations on aminoacylation activity of PoxA and AspRS. *Data taken from Eriani and Gangloff (11). *1 From α-Lysine experiments *2 From R-β-Lysine experiments(9). Residues highlighted in red are important for EF-P recognition.

Position mutated in PoxA from *E.coli*	Corresponding position in Yeast AspRS	kcat ratio mut/WT For Yeast AspRS*	Effect on amino acid activation	Mutant activity/ wildtype activity*1	k_{cat}/Km (μM^{-1} s^{-1})*2	k_{cat}/Km ratio mut/WT*2
H52A	Gap	-	Active	0.56	0.013	1.11
E103A	S329 (G73 + C74)	1.29	Active	<0.6	Inactive	---
R106A	T331 (G73)	0.61	Reduced	0.62	0.011	0.23
H108A	H334 (C74)	0.12	Active	<0.6	Inactive	---
D177A	Gap	-	Active	0.69	0.027	0.53
N180A	Gap	-	Active	0.52	0.041	0.82
E185A	Gap	-	Active	0.44	0.013	0.27
Q193A	D421	-	Active	0.75	0.05	1
S218A	E451	-	Active	0.96	0.047	0.93
R235A	S469	-	Reduced	<0.6	Inactive	---
E244A	E478	-	Inactive	0.04	Inactive	---
Wildtype				1		
neg				0.06		

Figure 2: Contact surface of Human LysRS/tRNA^{Asp} and *S. cerevisiae* AspRS/tRNA^{Asp}. Image corresponds to a superimposition of pdb files 1asy and 4dpg. *S. cerevisiae* AspRS is highlighted in red, Human LysRS in yellow and tRNA^{Asp} in purple. S469 residue in *S.cerevisiae* (blue) and R485 residue in Human LysRS (black) that corresponds to R235 in PoxA do not contact the tRNA. There is a distance of at least 9.14 Å between R485 from Human LysRS to tRNA.

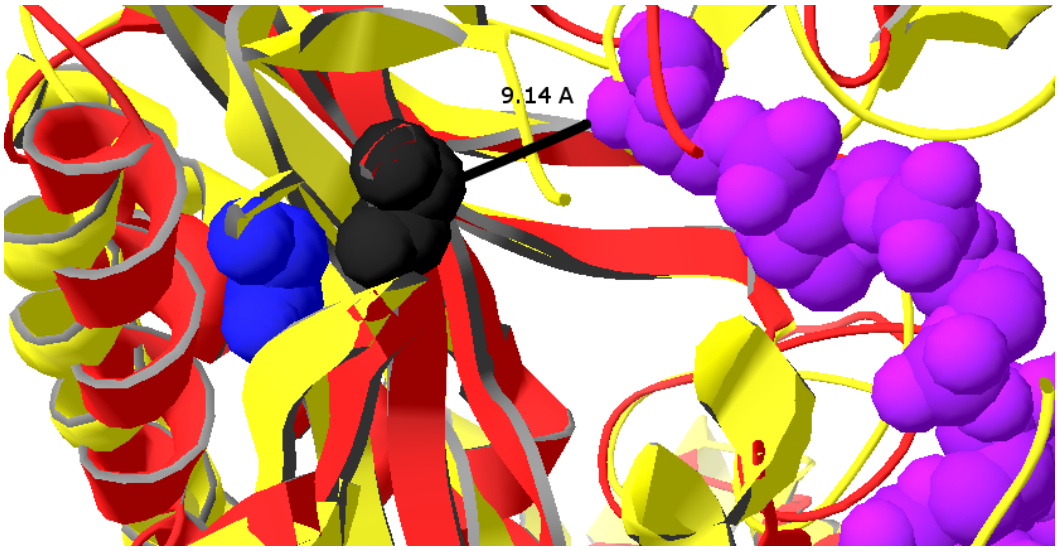

Figure 3: Mutating F29, K31 or G33 of EF-P to alanine blocks its recognition by PoxA.

Circles indicate wildtype EF-P, squares indicate the EF-P F29A mutant, diamonds are K31A and triangle is G33A

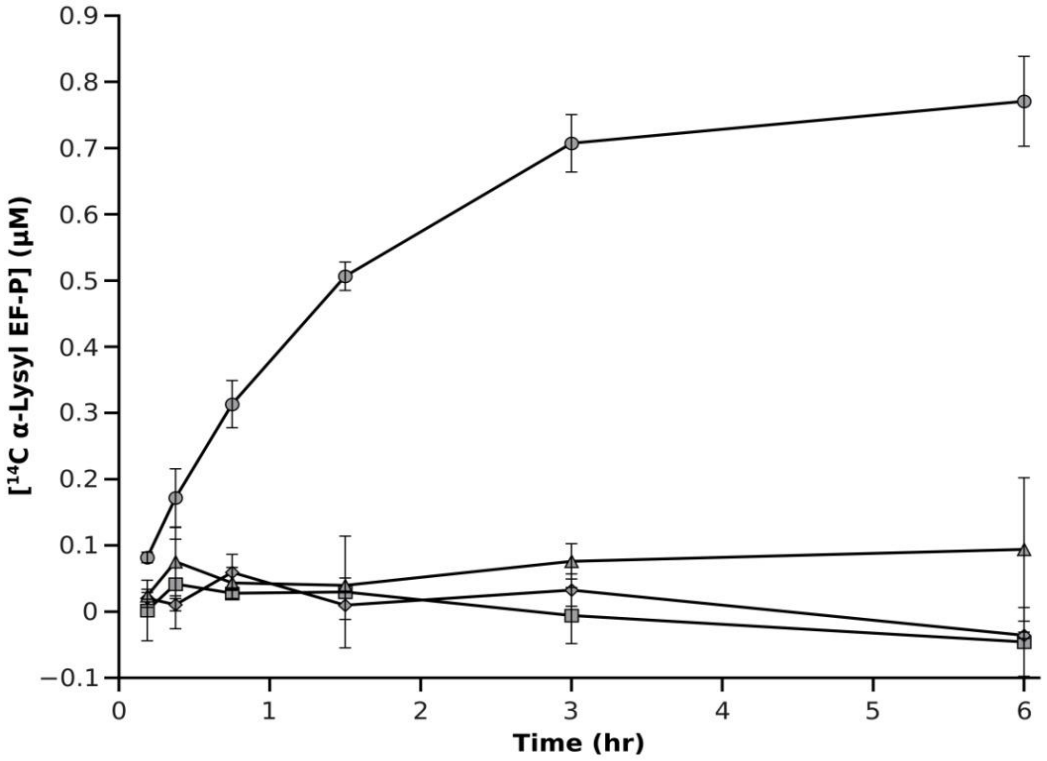

Figure 4: EF-P identity elements. Active site of PoxA. Green is EF-P, purple is PoxA. PoxA amino acids in which mutation abolished EF-P aminoacylation as well as the corresponding EF-P contacts are highlighted. These residues contact EF-P at the modification site.

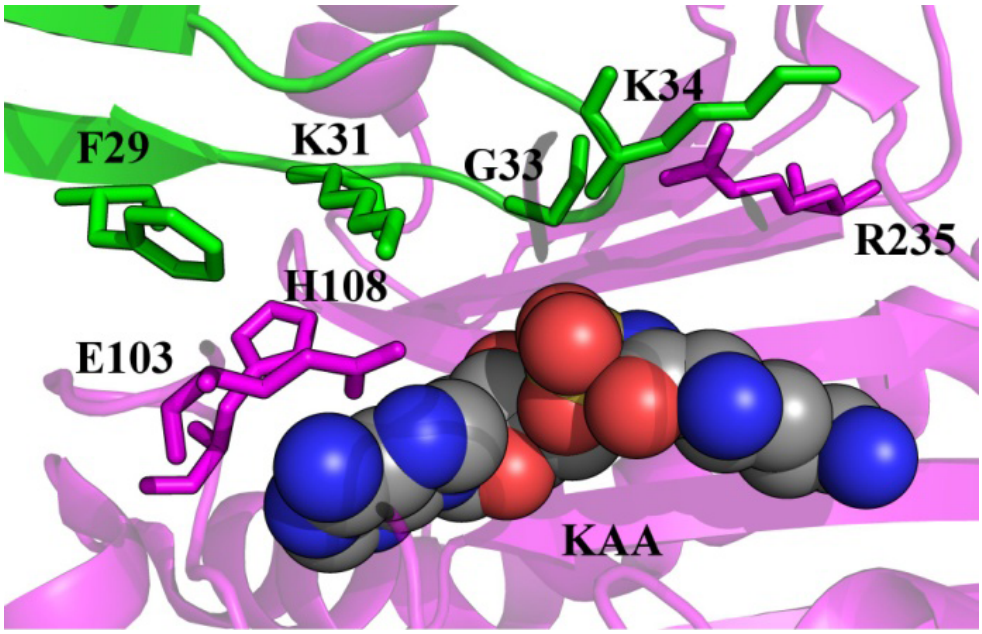

Figure 5: Alignment of amino acid sequences between synthetases. The amino acid responsible for the novel interaction that is important in EF-P recognition by PoxA (R235) R235 is only conserved in the Human Lysyl-tRNA synthetase. Corresponding amino acids are highlighted in red.

S. cerevisiae AspRS	467	SN**S**YDFFMRG-EEILSGAQRIHDHALLQE
Human AspRS	413	SN**S**YDMFMRG-EEILSGAQRIHDPQLLTE
Human AsnRS	459	TE**S**VDVLMPNVGEIVGGSMRIFDSEEILA
Human LysRS	483	TE**R**FELFVMK-KEICNAYTELNDPMRQRQ
E.coli PoxA	233	AE**R**FEVYYKG-IELANGFHELTDAREQQQ

Table 3. Primers used for PoxA mutagenesis.

Name	Sequence *[1]	Position mutated to alanine
mut_His52Ala_fd	GCGACGGTAACCGATATTgcTTTGGTCCCGTTTGAGACAC	His52
mut_His52Ala_rv	GTGTCTCAAACGGGACCAAAgcAATATCGGTTACCGTCGC	His52
mut_Glu102Ala_fd	CGCAGCTTCCGTAATGcAGAGATGGGGCGTTATC	Glu102
mut_Glu102Ala_rv	GATAACGCCCCATCTCTgCATTACGGAAGCTGCG	Glu102
mut_Glu103Ala_fd	CAGCTTCCGTAATGAAGcGATGGGGCGTTATCAC	Glu103
mut_Glu103Ala_rv	GTGATAACGCCCCATCgCTTCATTACGGAAGCTG	Glu103
mut_Arg106Ala_fd	CCGTAATGAAGAGATGGGGgcTTATCACAACCCTGAGTTCAC	Arg106
mut_Arg106Ala_rv	GTGAACTCAGGGTTGTGATAAgcCCCCATCTCTTCATTACGG	Arg106
mut_His108Ala_fd	GAAGAGATGGGGCGTTATgcCAACCCTGAGTTCACTATGC	Hist108
mut_His108Ala_rv	GCATAGTGAACTCAGGGTTGgcATAACGCCCCATCTCTTC	Hist108
mut_Asp177Ala_fd	GTCGCAGCGAAACTGGcTTTGAGCAATGTTGCTG	Asp177
mut_Asp177Ala_rv	CAGCAACATTGCTCAAAgCCAGTTTCGCTGCGAC	Asp177
mut_Asn180Ala_fd	CAGCGAAACTGGATTTGAGCgcTGTTGCTGATACCGAAGAAG	Asn180
mut_Asn180Ala_rv	CTTCTTCGGTATCAGCAACAgcGCTCAAATCCAGTTTCGCTG	Aspn180
mut_Glu185Ala_fd	CAATGTTGCTGATACCGcAGAAGACCGCGACACG	Glu185
mut_Glu185Ala_rv	CGTGTCGCGGTCTTCTgCGGTATCAGCAACATTG	Glu185
mut_Gln193Ala_fd	GACCGCGACACGCTGCTAgcATTGCTGTTTACCTTTGGC	Gln193
mut_Gln193Ala_rv	GCCAAAGGTAAACAGCAATgcTAGCAGCGTGTCGCGGTC	Gln193
mut_Ser218Ala_fd	GTACCACTTTCCAGCCgcCCAGGCATCACTGGCG	Ser218
mut_Ser218Ala_rv	CGCCAGTGATGCCTGGgcGGCTGGAAAGTGGTAC	Ser218
mut_Arg235Ala_fd	CGAAGATCATCGGGTCGCTGAAgcCTTTGAGGTTTATTATAAAGG	Arg235
mut_Arg235Ala_rv	CCTTTATAATAAACCTCAAAGgcTTCAGCGACCCGATGATCTTCG	Arg235
mut_Glu244Ala_fd	GGTTTATTATAAAGGTATTGcGCTGGCGAATGGTTTCCATG	Glu244
mut_Glu244Ala_rv	CATGGAAACCATTCGCCAGCgCAATACCTTTATAATAAACC	Glu244
mut_Arg303Ala_fd	GTGGCATTAGGTGTTGATgcTCTGGTGATGTTGGCGCTG	Arg303
mut_Arg303Ala_rv	CAGCGCCAACATCACCAGAgcATCAACACCTAATGCCAC	Arg303

*[1] Mutation site is marked in lower case.

Table 4. Primers used for EF-P mutagenesis.

Name	Sequence *[1]	Position mutated to alanine
mut_Phe29Ala_rv	CCTTTACCCGGTTTTACGGCTTCACTCGCTTCAACCGG	Phenylalanine 29
mut_Phe29Ala_fd	CCGGTTGAAGCGAGTGAAGCCGTAAAACCGGGTAAAGG	Phenylalanine 29
mut_Lys31Ala_rv	CCTGGCCTTTACCCGGTGCTACGAATTCACTCGCTTC	Lysine 31
mut_Lys31Ala_fd	GAAGCGAGTGAATTCGTAGCACCGGGTAAAGGCCAGG	Lysine 31
mut_Gly33Ala_rv	GCAATGCCTGGCCTTTAGCCGGTTTTACGAATTCAC	Glycine 33
mut_Gly33Ala_fd	GTGAATTCGTAAAACCGGCTAAAGGCCAGGCATTTGC	Glycine 33

*[1] Mutation site is marked in lower case.

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